Perspective

Making More MUCS

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Mucus protects, moisturizes, and lubricates mucosal surfaces. A child swirling mucus on his or her tongue has performed an experiment on its biophysical properties and is likely to be equally aware of the potential for dramatic increases in mucus, as with the common cold. However, too much of a good thing may be bad, and mucus hypersecretion may contribute to the pathophysiology of asthma, chronic bronchitis, and cystic fibrosis, as well as sinusitis and otitis media. Copious, hyperviscous mucus may directly obstruct airflow or impair mucociliary clearance. Stagnant mucus likely serves as both a nidus for bacterial colonization and perpetuates chronic infection.

Mucus hypersecretion is driven by mucous secretory cell hyperplasia and metaplasia, defined as more mucous secretory cells in proximal airways where they are usually found, and appearance in distal airways where they are normally absent, respectively. These phenotypic changes in the airway epithelium are hallmarks of chronic airway disease. Because increased mucus production is a common clinical complaint against which there are no specific therapies, and because improving mucociliary clearance may be beneficial, this is an area of great current interest.

Our understanding of the complex programs governing mucous secretory cell differentiation, enhanced secretory cell proliferation rates, increased expression of mucin genes, and hyperproduction and hypersecretion of mucin glycoproteins is incomplete, but current research is elucidating specific stimuli altering mucous secretory cells. As reviewed in Table 1, genetically manipulated mice provide new insights regarding mucous secretory cell biology. It is important to recognize that mice normally have few mucous secretory cells in their trachea, bronchi, and bronchioles that are instead dominated by a Clara cell lineage system (1). Conversely, in human beings, mucous secretory cells are found in the surface epithelium of many airway generations, down to the smallest 1 mm diameter bronchioles where there is a transition to a limited area of Clara cell–rich terminal bronchioles (2). Therefore, more mucous secretory cells in the mouse most likely represents metaplastic conversion of Clara cells into mucous cells, whereas in people, mucous secretory cell hyperplasia is predominant. Thus, the valuable lessons from mice must be translated to human cells and, ultimately, human beings.

The article by Vermeer and colleagues (3) in this issue of the *AJRCMB* is an example of an *in vitro* study using human tracheobronchial epithelial cells cultured at air–liquid interface conditions that promote mucous and ciliated cell differentiation resembling that found *in vivo* (Figure 1). The authors make the important point that interleukin (IL)-9 stimulation of mucous secretory cell hyperplasia is context-dependent. When IL-9 was added to mature, well differentiated cultures there was apparently little effect, but if IL-9 was added to actively differentiating or wounded cultures there was an increase in the number of mucous secretory cells and in secreted mucous. The authors speculate that the combination of elevated IL-9 and pathologic epithelial turnover may underlie the mucus hypersecretory phenotype in asthma. In contrast, IL-13 alone, without injury, increased mucous secretory cells. A handful of other studies using similar human cultures have examined the stimulation of mucous secretory cells (Table 2). These studies highlight the technical and experimental challenges associated with quantifying mucus and mucous cells in this most relevant of models. The main purpose of this short perspective is to discuss key elements of analyzing the mucus secretory apparatus in well differentiated human cell cultures. Similar principles will hold in attempts to measure mucins in human lavage fluid, expectorated sputum, and biopsies.

Human Airway Epithelial Cell Culture Sample Size

Since the breakthrough study of Whitcutt, Adler, and Wu (4), the gold standard for obtaining well-differentiated airway epithelial cultures is to grow cells on a porous support at an air–liquid interface. This method is readily applicable to human cells as indicated by studies too numerous to mention in this brief Perspective. Yet, these cultures are much more challenging than conventional cells on plastic. Limited numbers of labs procure their own human airway cells. Commercially available human airway cells are prohibitively expensive for many labs, and cells from only one or a few individuals may be available at any given time. Each batch of primary passaged cells will have distinct growth and differentiation capacities. Polymorphisms in the human population affecting cell growth, differentiation, and response to stimuli as well as technical variability between

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Abbreviations: interleukin, IL; messenger RNA, mRNA; reverse transcriptase–polymerase chain reaction, RT-PCR;

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TABLE 1
A partial list of cytokine/chemokine regulation of mucous secretory cells in genetically manipulated mice

Factor	Effect	References
IL-4	Transgenic mice expressing IL-4 in the lung show lung mucus accumulation and hypersecretion, mucus cell metaplasia and increased *mMuc5ac* expression	(14–16)
IL-5	Transgenic mice expressing IL-5 in the lung show mucus accumulation and mucus cell metaplasia in the airways	(17)
IL-9	Transgenic mice expressing IL-9 in the lung show airway mucus accumulation and increased *mMuc2* and *mMuc5ac* expression	(18–20)
IL-10	Transgenic mice overexpressing IL-10 show mucus cell metaplasia	(21)
	IL-10-deficient mice show reduced mucus production in response to lung insult	(22)
IL-11	Transgenic mice overexpressing IL-11 in the lung show mucus hypersecretion and mucus cell metaplasia	(23)
IL-13	Transgenic mice overexpressing IL-13 in the lung show mucus hypersecretion and mucus cell metaplasia	(24, 25)
	IL-13-deficient mice show reduced mucus hypersecretion after acute lung insult	(26)

Definition of abbreviation: IL, interleukin.

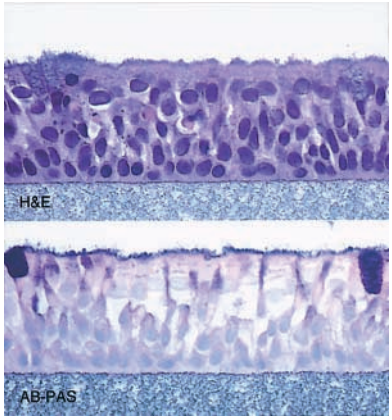

Figure 1. Paraffin sections of formalin-fixed, passage 1 human tracheobronchial epithelial cells grown in culture at an air–liquid interface for 21 d. The porous support is visible below the cells. Mucous secretory cell granules as well as cell surface materials are easily visualized in the *lower panel*. *H&E*, hematoxylin and eosin stain; *AB-PAS*, alcian blue–periodic acid-Schiffs stain. *Original magnification*, 400×.

different lots of cells will cause variability and dictate larger sample sizes. This raises the important issue of definition of sample number. Do 20 replicate cultures from one individual represent an *n* of 20? Careful studies of mucus hypersecretion will require consistently differentiated cultures from a representative population of humans. As a minimum, we suggest that the old laboratory axiom of "three times in triplicate" is applicable, where three times represents a minimum of cultures from three different individuals, and each experimental group consists of three replicate cultures. In practice, many parameters will require much larger sample sizes. It is important for authors to clearly present their working definitions of sample size.

Defining and Measuring Mucus

Respiratory tract mucus consists of biomolecules, ions, and water, including the highly glycosylated, polymeric, secreted, gel-forming mucins MUC5AC, MUC5B, and, on occasion, MUC2. Also present are highly glycosylated, monomeric MUC1 and MUC4 mucins shed from the cell surface as well as additional N and/or O-glycosylated proteins, lipids, and glycolipids. The complex and variable nature of mucins and their carbohydrate modifications present challenges for their quantitation.

Mucin Gene Expression

The gel-forming mucins predominantly determine mucus viscoelastic and adhesive properties. Thus, standard quantitative methods for assessing MUC2, MUC5AC, and MUC5B messenger RNA (mRNA) abundance, such as northern blots, nuclease protection assay, and quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) are particularly useful tools for analyzing cultures. Recent advances in the sequencing of mucin genes facilitates this task and also enables analysis of MUC1 and MUC4, as well as any newly discovered or less well-studied mucins. As discussed below, measuring mucin mRNA is more straightforward than analyzing mucin glycoproteins. However, measurement of mucin mRNA was not exploited by Vermeer and colleagues (3). It is notable that Northern blot and nuclease protection require more RNA than does RT-PCR. Quantitative RT-PCR, using optimized and specific primers, will likely find great use in the future. Promoter sequences of MUC5AC and MUC5B have been defined (5, 6), and promoter activity can be readily assayed in cell lines by expressing a reporter gene under the control of mucin promoters (7). However, due to the low transfectability of well-differentiated airway epithelial cells with routine plasmid protocols, this strategy is not yet feasible. Adenoviral vectors combined with methods to enhance access to the basolateral membrane (8) offer promise for future promoter–reporter studies. For MUC2, MUC5AC, and MUC5B, this will rely on adequate transfection of mucous secretory cells.

Quantitating Mucin Glycoproteins

Ultimately, it will be important to quantitate production and secretion of mucin glycoproteins. The approach of metabolic labeling with 3H glucosamine followed by apical harvest of secretions, enzyme digestion, gel filtration, and density gradient purification (9, 10) is a time-tested but unwieldy standard. This has led to a search for more convenient assays. However, this is difficult for several reasons. First, mucus varies from a viscous fluid to a strongly viscoelastic gel, not dispersable in physiological solutions. Dispersion is achieved using chaotropes such as 4–6 M guanidinium chloride combined with reducing agents such as dithiothreitol. While effective for dispersion, such treatment inhibits the use of the sample in simple biochemical assays. Within the resultant mixture of large and small molecules, smaller, more dynamically active species will blot on mem-

TABLE 2
Partial list of cytokine/chemokine regulation of mucous secretory cells in well-differentiated human airway epithelial cell cultures.

Factor	Effect	References
IL-4	Decreases *MUC5AC* expression and MUC5AC secretion in normal HNE cells	(27)
	Increases *MUC8* expression in normal HNE cells	
	Decreases *MUC5AC and MUC5B* expression in HBE cells	(28)
IL-9	Increases *MUC2* and *MUC5AC* expression in normal HBE cells	(19, 29)
IL-13	Increases the number of mucous cells in normal HNE cells	(30)
	Decreases *MUC5AC* expression in normal HNE cells	(31)
	Increases *MUC2* and *MUC8* expression	

Definition of abbreviations: HBE, human bronchial epithelial; HNE, human nasal epithelial; IL, interleukin.

branes or bind to ELISA plates more efficiently, resulting in under-representation of mucin. One solution is a capture assay, such as a sandwich ELISA. However, if the sample required dispersion, chaotropes or reducing agents must be removed or decreased to avoid denaturing the capture reagent, at the risk of sample aggregation or insolubility. One answer is agarose electrophoresis to mostly purify smaller molecules away from the large mucins followed by vacuum blotting to a membrane and subsequent identification with a variety of suitable probes (11). While the process is fairly simple, it is not trivial and requires a well-characterized mucin standard. It is also optimal to use specific probes, such as antibodies to the gel-forming mucins. When surrogate markers such as lectins are used, it must be appreciated that they promiscuously identify specific terminal sugars on glycolipids or N- or O-linked oligosaccharides. The sugar residues may be cryptic or over-represented, depending on factors such as blood group or physiologic state of the particular sample. Clear association of the surrogate probe with the relevant mucins is required.

Morphologic Approaches to Quantitating Mucous Cells

Human airway epithelial cells in culture develop a mucociliary morphology consisting of basal, ciliated, and mucous secretory (goblet) cells after 14–21 d at an air–liquid interface. Their characteristic morphology and chemistry distinguishes apically oriented goblet cells, making them amenable to several light or electron microscopic morphometric techniques. These include determining volume density of stored alcian blue–periodic acid-Schiff–positive materials, estimation of goblet cell density per millimeter basal lamina, and the thickness of apical secretions when specially preserved with perflurocarbon osmium tetroxide fixation (12). The latter two approaches were used by Vermeer and colleagues (3). In addition, these authors used a surrogate marker, fluorescein isothiocyanate–labeled jacalin lectin, viewed by confocal microscopy. In parallel experiments, jacalin lectin mostly colocalized with an anti-MUC5AC monoclonal antibody. As for the biochemical measurements discussed above, it is important to recognize that terminal sugar residues may vary based on human polymorphisms and the metabolic state of the cell. Thus, lectins should be viewed as risky surrogate markers. Additionally, rigorous morphometry is predicated on solid methodologic principles, including adequate, unbiased sampling, clear structural definitions, and special considerations for enumeration of variably sized objects or oriented samples as previously outlined for studies of the lung (13). Similar to the sample size definitions noted above, it is important for authors to clearly present the details of the morphometric analysis.

Conclusion

The pulmonary community is entering an era in which we may satisfy our child-like fascination with mucus. We will likely uncover key aspects regulating the quantity, quality, and secretion of mucus, expanding our knowledge of its role in normal physiology and in disease. Additional studies such as those of Vermeer and colleagues (3), using well-differentiated primary human airway epithelial cells and incorporating future improvements in our analytic capacity of the mucus secretory apparatus, will hopefully suggest novel therapies beneficial to our fight against airway disease.

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